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TITLE: Reversing Maladaptive Plasticity to Cure Autonomic Dysreflexia after Spinal Cord Injury

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CONTRACTING ORGANIZATION: The Ohio State University, Columbus, OH 43210

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Fort Detrick, Maryland 21702-5012

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14. ABSTRACT Autonomic dysreflexia (AD) is a potential life threatening condition characterized by episodic vascular hypertension (often with bradycardia) that develops in most people with a spinal cord injury (SCI) above thoracic spinal level T5. Using telemetric recording we were able to detect biphasic spontaneous AD developed in mice with T3 SCI; the early phase of AD occurs within first week which is likely due to loss of descending control of sympathetic outflow and the late phase occurs weeks post injury which is likely caused by the formation of aberrant sympathetic neural circuits at the site of injury. We proposed that post-injury inhibition of reactive synaptogenesis would block the onset or reduce the severity of delayed onset AD. Experiments funded by this grant will test this hypothesis using both genetically modified mice ($\alpha 2\delta$-1 KO, $\alpha 2\delta$-1 over-expressing and TSP KO) and wild-type mice receiving chronic infusions of Gabapentin (GBP) – a drug that inhibits binding of neuronal $\alpha 2\delta$-1 receptors with glial thrombospondins (TSP). We predict that chronic GBP will block the formation of aberrant sympathetic nerve circuits and prevent the onset of AD. Similar results are predicted using mice deficient in either the $\alpha 2\delta$-1 receptor or TSP. Conversely, transgenic overexpression of $\alpha 2\delta$-1 is expected to accelerate the onset of AD or exacerbate severity of dysreflexia. Preliminary data generated in Year 1 show that the frequency of AD increases early after SCI in mice engineered to overexpress $\alpha 2\delta$-1; however, these effects are transient -- late phase AD does not develop in transgenic mice or in mice with the genetic background on which these transgenics were created. Future studies will determine AD frequency in the knockout mice (see above) or C57BL/6 mice treated with GBP.					
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INTRODUCTION

Autonomic dysreflexia (AD) is a life threatening condition of episodic vascular hypertension (often with bradycardia, i.e., slowed heart rate) that develops in most people with a spinal cord injury (SCI) above thoracic spinal level T5. After high level SCI, loss of supraspinal control together with aberrant collateral sprouting and formation of new intraspinal synapses causes spinal autonomic reflexes to become exaggerated [1-4]. This post-injury maladaptive neural plasticity, involving sensory axons and propriospinal interneurons that connect multiple segments of the thoracic and upper lumbar spinal cord, progresses slowly over the course of several weeks or months post-injury. Prevention (e.g., regular bladder/bowel care) and anti-hypertensive medications are currently the best way to “manage” AD; however, there is no cure [5]. In this proposal, genetic and pharmacological tools will be used to test the hypothesis that post-injury inhibition of reactive synaptogenesis will block the onset or reduce the severity of AD.

After CNS injury, astroglia and macrophages secrete thrombospondins (TSP), a family of matricellular proteins that regulate cell-cell and cell-matrix interactions, most notably neurite growth and synaptogenesis [6, 7]. Eroglu et al. showed that astrocyte-derived TSPs cause synaptogenesis by binding to neuronal $\alpha 2\delta$ -1 receptors and that transgenic over-expression of neuronal $\alpha 2\delta$ -1 dramatically increases synaptogenesis [7]. TSP-4 is selectively increased in astrocytes surrounding injured spinal cord axons. Studies in this proposal will test that hypothesis that genetic deletion of either TSP-4 or $\alpha 2\delta$ -1 will block the onset or reduce the severity of AD; however over-expression of $\alpha 2\delta$ -1 will enhance AD development. The anti-epileptic/anti-neuropathic pain drugs gabapentin (Neurontin) and pregabalin (Lyrica) bind with $\alpha 2\delta$ -1 [8] thereby blocking TSP/ $\alpha 2\delta$ -1 interactions and subsequent formation of new synapses [7]. We hypothesize that chronic infusions of GBP will inhibit TSP binding to neuronal $\alpha 2\delta$ -1 which will subsequently inhibit maladaptive synaptogenesis and reduce the severity and frequency of AD.

During the first year of funding, significant progress was made on 3 of 4 tasks designated as part of Aim 1. First, colonies of two different genetically engineered mouse strains were established at Ohio State – an $\alpha 2\delta$ -1 over-expressing transgenic mouse line and a separate line of TSP-4 null mutant mice. Second, we modified our published algorithm that allowed semi-automated detection of spontaneous AD in awake, freely moving mice. This new algorithm was compared with the old algorithm. Importantly, an independent investigator reproduced our original published data and verified that the new algorithm is robust but is much more efficient. Finally, using the new algorithm, new preliminary data indicate that the frequency of spontaneous AD increases in $\alpha 2\delta$ -1 over-expressing mice; however, these effects are transient and late-phase AD does not occur spontaneously nor can it be induced in transgenic mice or wild-type littermate control mice (129S2/SvPasCrl background). These data, although preliminary, indicate that there is a genetic predisposition to the onset and maintenance of AD. This is a novel observation. We will expand on these experiments in year 2 and will also begin testing whether the onset and development of AD is affected in mice deficient in $\alpha 2\delta$ -1 or TSP-4. The former null mutant mouse line is currently being bred/maintained by our collaborator, Dr. Cagla Eroglu, at Duke University.

KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Spinal cord injury (SCI), Autonomic dysreflexia (AD), Thrombospondin (TSP), $\alpha 2\delta$ -1 receptor, Gabapentin (GBP).

ACCOMPLISHMENTS:

What were the major goals of the project?

[from approved Scope of Work]

Aim 1: Use genetic tools to reduce maladaptive post-injury synaptogenesis and the frequency and/or severity of AD.

- Task 1: Set-up breeding colonies for TSP-4 null mice and $\alpha 2\delta$ -1 transgenic mice (Months 1-4).

*Milestone 1: Establish colony of **TSP-4 null mice** and **$\alpha 2\delta$ -1 transgenic mice** at OSU.*

[completed]

- Task 2: Complete SCI surgeries on TSP-4 null mice and $\alpha 2\delta$ -1 transgenic mice then monitor autonomic dysreflexia using telemetry (Months 5-10).

Milestone 2: Complete in vivo experiments on two (of three) mouse lines. Order of completion will depend on breeding success and mouse availability. However, we will not mix/match mouse colonies within an experiment, i.e., either TSP-4 null mice OR $\alpha 2\delta$ -1 transgenic mice will be compared to their respective controls in a single study. [~15% complete]

- Task 3: Analyze telemetry data from Task 2 (Months 11-13). *[in progress]*
- Task 4: Complete histological processing, immunostaining and synapse quantification for TSP-4 null, $\alpha 2\delta$ -1 transgenic and littermate control mice (Months 13-21). *[in progress]*

Milestone 3: Prepare data from TSP-4 null mice and $\alpha 2\delta$ -1 transgenic mice for presentation at national meeting (e.g., Society for Neuroscience). [in progress]

What was accomplished under these goals?

1) Major activities and specific objectives:

- **Completed Task 1 - established two new mouse colonies at Ohio State.**

We obtained $\alpha 2\delta$ -1 transgenic (TG) and TSP-4 null mutant (KO) breeding pairs from Dr Eroglu (Duke) on Feb 7, 2014. Mating was initiated after clearing quarantine and by the middle of August (2014), enough mice (n=10 for $\alpha 2\delta$ -1 TG and n=9 for WT littermates) were available to begin experiments (see Task 2).

- **We revised our previously published algorithm that allowed semi-quantitative analysis of spontaneous AD. In an effort to improve the efficiency and reliability of telemetry data, a new algorithm was developed. Using this new algorithm, a new post-doc in the lab independently verified the temporal sequence of spontaneous AD after high level SCI in C57BL/6 mice. The new algorithm then was used to begin experiments described for Aim 1.**

Dataquest data acquisition software (Data Sciences International) was used to acquire continuous HR and BP data between 5 and 35 dpi at 5s intervals. MATLAB software was used to create an algorithm that would detect episodes of spontaneous AD. This new algorithm works similarly to a previous semi-automated method that we developed [9] but it improves objectivity and efficiency (see **Figure 1**, below). The new algorithm initially screens events by checking for segments of data that meet the time (T1, T2) and curve height (H1, H2) parameters. T1 and T2 intervals are defined by the intersection of the blood pressure and heart rate curves, respectively, with their baseline curves. If these parameters are met, the T3 bounds are set using a 30 second lowess smoothing window. The short time window allows extraneous peaks to be removed without removing localized changes from the data. The onset of the decrease in heart rate is defined as the first local maximum of the 30 s smoothing window before the local minimum heart rate during the event. Similarly, the end of this decrease is defined as the first local maximum after the local minimum of the event. Together, these bounds mark the T3 boundaries. The time values associated with these boundaries are used to find similar boundaries for the blood pressure data. The minimum blood pressures values within 20 seconds of the T3 boundaries mark the onset and end of the blood pressure increase. From these values, the diagonal line is set, which connects the boundary points of the blood pressure increase. This line is used to calculate the H3 parameter, which must be at least 20 mmHg vertically from the line to the blood pressure curve. H1, H2, and H3 can occur anywhere between the bounds of the event (T1 for blood pressure, T2 for heart rate). After all parameters have been evaluated for a potential event, and the event has been classified as AD, the event is plotted and saved for any later visual confirmation that may be needed. To validate the automated algorithm, the same data was analyzed using both the semi-automated and fully automated techniques. The results are shown below in Figure 1C. The two methods yield similar results, with the fully automated technique typically detecting more false-positive events than the semi-automated technique. The trade-off is that the automated detection is completely unbiased and significantly more efficient, requiring ~80% less time to complete analysis.

- **Completed part of Tasks 2&3. Specifically, in vivo telemetry experiments were completed to measure the onset and frequency of spontaneous AD using $\alpha 2\delta$ -1 null or 129/sve littermate wild-type (control) mice. All were subjected to high-level (T3) SCI. The telemetry data were analyzed and tissues prepared then shipped to Dr. Eroglu at Duke University for analysis of synapses (as described in Task 4).**

Telemetry transmitter implantation: All animals were maintained in a pathogen-free environment before surgically implanting telemetry transmitters. Telemetry transmitters were implanted into each mouse as described previously [9]. Briefly, the PhysioTel telemetry system with PA-C10 transmitters (Data Sciences International) were implanted in anesthetized mice 3 days before SCI via a cannulation of the left common carotid artery (CCA) to monitor the blood pressure (BP)

and heart rate (HR). The extra-vascular portion of the transmitter was placed into a subcutaneous pocket created on the lateral flank. The CCA was exposed through a midline incision on the neck, and the catheter of the transmitter was inserted into the CCA through a small incision near the carotid bifurcation then advanced until the sensing region of the catheter was positioned in the aortic arch (~8–9 mm from the carotid bifurcation).

Spinal cord injury: Complete T3 spinal cord transection injuries were performed as described previously [10]. Briefly, using aseptic technique, a partial laminectomy was performed at vertebral level T3, after which the periosteum and dura mater were carefully opened. Using iridectomy scissors together with gentle aspiration, the spinal cord was cut, creating a clear separation between the rostral and caudal stumps of transected spinal cord. After injury, muscle and skin were sutured separately and then mice were injected with sterile saline (2 ml, s.c.) then placed individually into warmed HEPA filtered cages. Postoperative care included manual bladder expression (at least twice daily) with daily antibiotics (gentocin, 5mg/kg, s.c.) for the first 7 days post-injury (dpi). Dehydration was monitored daily while body weight and urinary pH were monitored weekly. All surgical procedures were approved by and performed in accordance with the Institutional Laboratory Animal Care and Use Committee at The Ohio State University.

Analysis of spontaneous AD: See above (and Fig. 1, below).

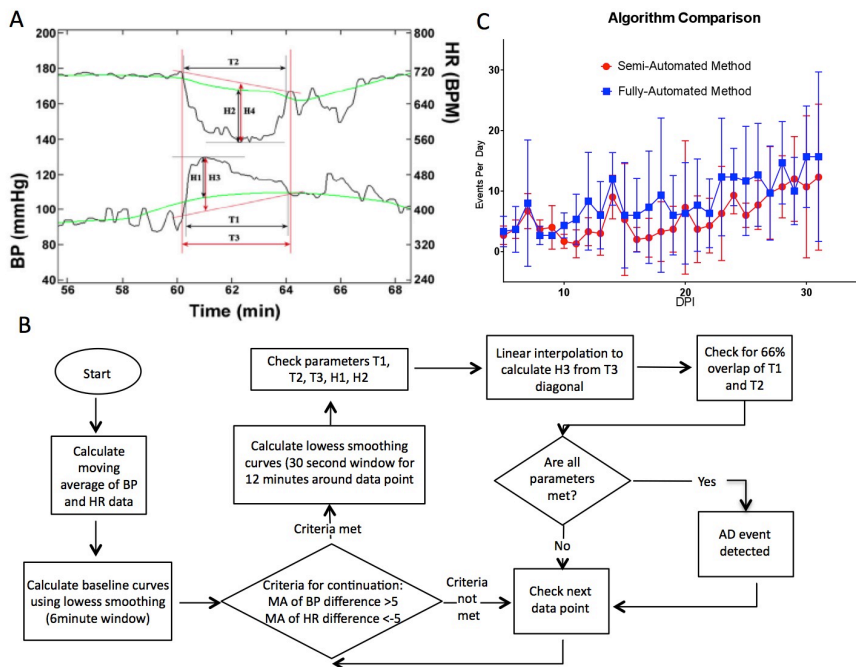


Figure 1. Fully automated MATLAB algorithm designed to detect spontaneous AD in SCI mice. For the program to detect a “dysreflexic event,” a BP change from baseline (H1) must exceed 10 mmHg and persist above baseline for > 30s (T1). During T1, HR must decrease at least 10 bpm below baseline (H2). Moreover, the duration of the BP increase (T1) and HR decrease (T2) must overlap for at least 66% of the measured interval. H3, T3 and H4 parameters, which were originally completed by hand, were incorporated into the algorithm. Only spontaneous AD events in which H3>20 mmHg, T3>30 s and H4>10 BPM were tallied and plotted. (A). Flow chart for automatic AD detection. (B). Comparison between the result generated by semi-automated (old) and fully-automated (new) AD detection method. (C)

Eliciting AD via colorectal distension or cutaneous pinch: Colorectal distension or cutaneous pinch were used to elicit spinal autonomic reflexes as described previously [9]. The tips of Hartman hemostats were shielded with polyethylene tubing and then used to pinch the flank below the level of SCI just rostral to the hip joint. To ensure consistent pinch intensity and duration, the hemostat was closed to the first click in every trial for 30s. Colorectal distension was accomplished using a 4-French, 60

mm balloon-tipped catheter (Swan-Ganz monitoring catheter model 116F4; Edwards Life Sciences). The catheter was inserted into the anus, positioning the balloon ~1.5 cm from the anal opening and then securing the catheter to the tail with surgical tape. After securing the catheter, animals were left alone to acclimate for at least 20 min. To elicit AD, the balloon was inflated with 0.3 ml of air for 1 min. Distention was maintained for 1 min and repeat stimulation occurred after a 30 min rest. Peak changes in BP and corresponding HR were obtained and then compared with baseline values.

2) Significant results or key outcomes

New, more efficient AD detection algorithm: Automated detection of AD events was established using a MATLAB program and validated through comparison with the semi-automated techniques (**Figure 1**). Overall, the new and fully automated algorithm works similarly to the previous semi-automated method, but incorporates the manual validation steps into the computer algorithm to improve objectivity and efficiency.

In vivo analysis of AD in SCI a2d1 transgenic mice: By 5dpi, there is a significant increase in spontaneous AD in T3 SCI a2d1 TG mice. These data are consistent with our previously published data using C57BL/6 mice [9]. However, the frequency of AD in littermate controls (on 129 background) is low and is significantly less than in a2d1 Tg mice during the first week post-injury (**Figure 2**).

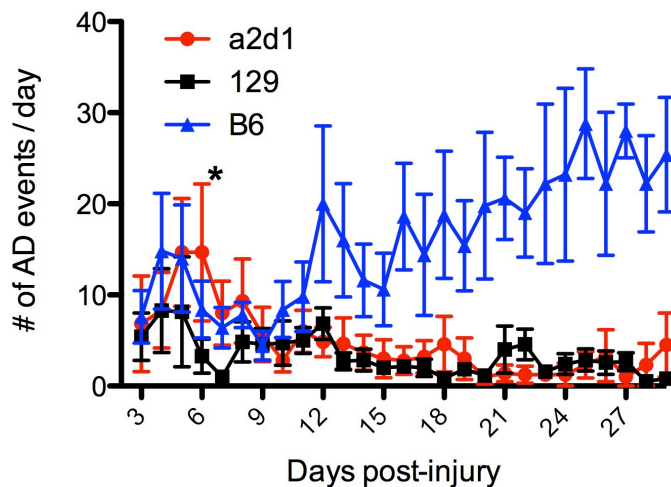


Figure 2. Spontaneous AD develops early, but not later, after SCI in a2d-1 transgenic (TG) mice. The frequency of AD events is similar to what is observed in T3 SCI C57BL/6 (B6) mice. Late phase spontaneous AD does not develop in TG or 129 wild-type littermate control mice. n=4-7/group; * p<0.05

Importantly, neither a2d1 TG nor WT littermate control mice develop late-phase AD. This is in contrast to what occurs in C57BL/6J (B6) mice with an identical SCI. These data, although preliminary, indicate that there is a genetic predisposition to the onset and maintenance of AD. This is a novel observation.

To further challenge the spinal autonomic circuitry and unequivocally determine whether AD can be elicited in SCI mice on the 129 background, mice were challenged with noxious visceral or cutaneous stimuli. To elicit spinal autonomic reflexes, skin pinch (below the level of injury) or colorectal

distension were used and HR and BP recorded as described previously [9]. The data indicate that it is not possible to elicit AD in 129 mice with high-level SCI (~80%) of those animals (**Figure 3**).

Previously, we generated data in mice and humans implicating episodic autonomic dysreflexia as a mechanism underlying systemic immune suppression after SCI [9]. In mice, AD-induced immune suppression is consistently associated with splenic atrophy [9]. Since mice on a 129

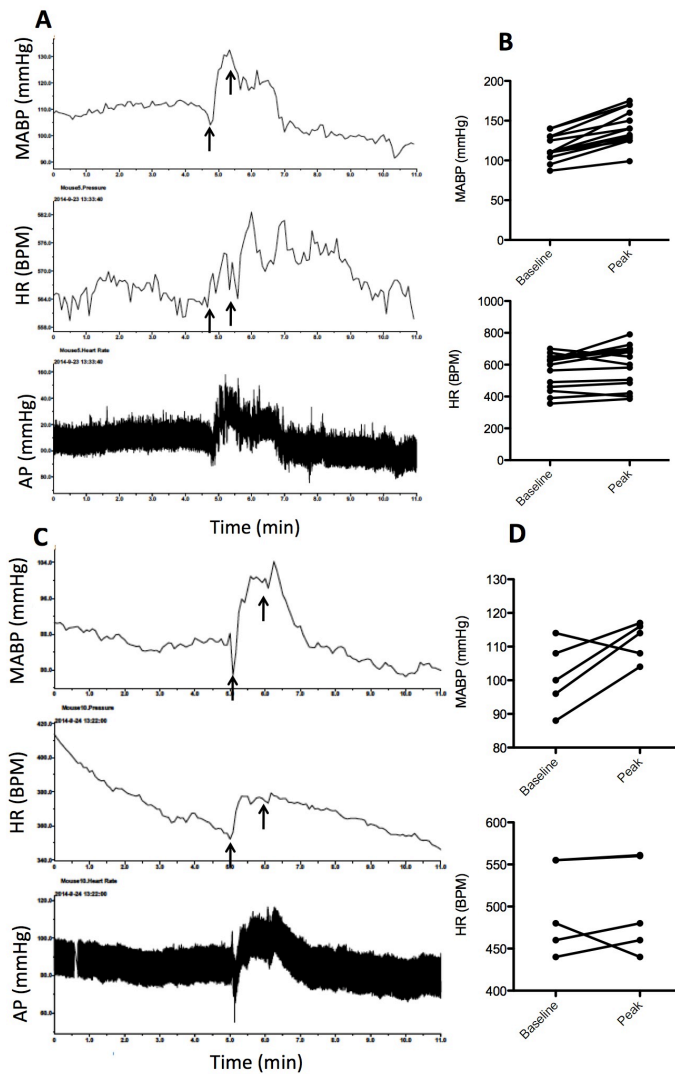


Figure 3. Neither cutaneous pinch nor colorectal distension (CRD) elicit AD in 129 mice. Representative pulsatile arterial pressure traces after applying cutaneous pinch (A) or CRD (C) in mice at 35dpi. Changes in MABP and mean HR were quantified for cutaneous pinch (B) or CRD (D). Arrows on each trace indicate the onset and termination, respectively, of the stimulus. n=4/group; *p<0.05 versus baseline.

3) Other achievements.

Nothing to report.

What opportunities for training and professional development has the project provided?

Nothing to Report

background do not develop AD, we asked if splenic atrophy occurred in these mice. Spleens of from a2d1 TG or WT mice were isolated 38 days post T3 SCI and then wet weights were obtained then normalized to post-injury body weight. Despite the absence of AD, splenic atrophy is a consistent phenomenon after T3 SCI in both a2d1 TG and WT 129 mice (Figure 4).

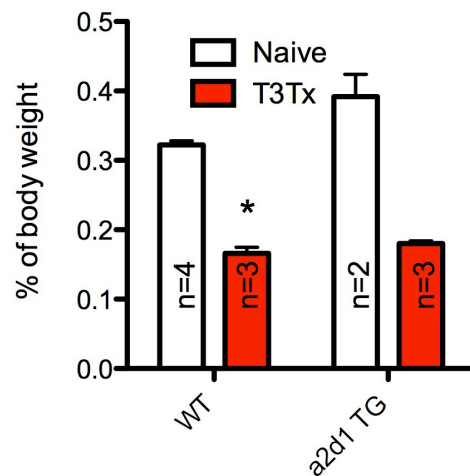


Figure 4. Spleen weight is significantly reduced at 38 dpi in both WT and a2d1 TG mice n=3-4 for WT and n=2-3 for TG; *p<0.01.

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

For the next reporting period, we plan to repeat the experiment described above comparing a2d1 TG vs WT mice. We also anticipate being able to complete one experiment involving TSP4 KO mice. Completing these goals would put us ahead of schedule for completing four of six milestones within two years.

Also in year 2, we will begin to characterize the bio-distribution and intraspinal concentrations of GBP after daily infusions for 28 days (Aim 2).

IMPACT:

What was the impact on the development of the principal disciplines of the project?

The new preliminary data suggest that the development of sustained AD is eliminated or markedly reduced in 129 mice. From these data we hypothesize that genetics play a previously unappreciated role in whether AD develops after high-level SCI. Indeed, it is possible that the neural plasticity that influences autonomic dysfunction after high-level SCI is influenced by genetic determinants. To my knowledge, this has never been described in the context of autonomic function after SCI; however, there are published data showing that genetics can influence autonomic control of glucose metabolism. Whether genetics also modulate neural plasticity in the autonomic nervous system and subsequent control of the cardiovascular response is not known.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

CHANGES/PROBLEMS:**Changes in approach and reasons for change**

Nothing to report

Changes that has a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Significant changes in use or care of human subjects

NA

Significant changes in use or care of vertebrate animals

NA

Significant changes in use of biohazards and/ or select agents.

NA

PRODUCTS

Publications, conference papers, and presentations

Nothing to report

Website or other Internet site

Nothing to report

Technologies or techniques

The MATLAB algorithm described in this report has been created specifically to meet the needs of this funded research program. To our knowledge, this is the only automated algorithm for detecting AD in SCI mice. Pending further (independent) validation of the algorithm, we plan to publish the algorithm.

Invention, patent applications, and/or licenses

Nothing to report

Other products

Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Yan Wang
Project Role:	Postdoc Researcher
Researcher Identifier	200143183
Nearest person month worked	12
Contribution to project:	Performs experiments, data analysis, writing
Funding support:	
Name:	Zhen Guan
Project Role:	Senior Research Associate
Researcher Identifier	
Nearest person month worked	12
Contribution to project:	Performs experiments (surgery; animal care)
Funding support:	
Name:	J. Hayes Davis
Project Role:	Undergraduate Researcher
Researcher Identifier	2003187666
Nearest person month worked	10
Contribution to project:	data analysis (algorithm design)
Funding support:	
Name:	Phillip Popovich
Project Role:	Professor
Researcher Identifier	
Nearest person month worked	12
Contribution to project:	Designs the experiments, data analysis, writing
Funding support:	

Has there been a change in the active other support of the PI or key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Dr. Cagla Eroglu, Ph.D.

Organization Name: Duke University Medical Center Department of Cell Biology

Location: 334 Nanaline Duke Building, Durham, NC 27710

SPECIAL REPORTING REQUIREMENTS

Collaborative awards:

For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award

QUAD CHARTS:

If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.

APPENDICES

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc

1. Weaver, LC, Fleming, JC, Mathias, CJ, and Krassioukov, AV. Disordered cardiovascular control after spinal cord injury. *Handb Clin Neurol* 109: 213-233.
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